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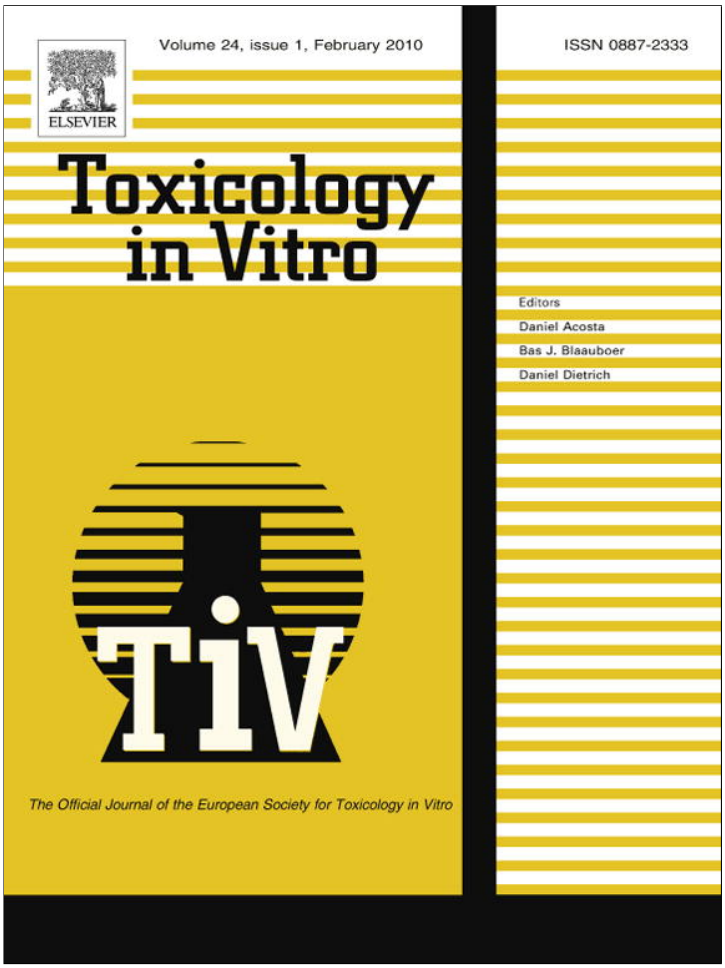

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Comparative *in vitro* and *ex-vivo* myelotoxicity of aflatoxins B1 and M1 on haematopoietic progenitors (BFU-E, CFU-E, and CFU-GM): Species-related susceptibility

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ABSTRACT

Haemato- and myelotoxicity are adverse effects caused by mycotoxins. Due to the relevance of aflatoxins to human health, the present study, employing CFU-GM-, BFU-E- and CFU-E-clonogenic assays, aimed at (i) comparing, *in vitro*, the sensitivity of human *vs.* murine haematopoietic progenitors to AFB1 and AFM1 (0.001–50 μg/ml), (ii) assessing whether a single AFB1 *in vivo* treatment (0.3–3 mg/kg b.w.) alters the ability of murine bone marrow cells to form myeloid and erythroid colonies, and (iii) comparing the *in vitro* with the *in vitro ex-vivo* data.

We demonstrated (i) species-related sensitivity to AFB1, showing higher susceptibility of human myeloid and erythroid progenitors (IC_{50} values: about 4 times lower in human than in murine cells), (ii) higher sensitivity of CFU-GM and BFU-E colonies, both more markedly affected, particularly by AFB1 (IC_{50}: 2.45 ± 1.08 and 1.82 ± 0.8 μM for humans, and 11.08 ± 2.92 and 1.81 ± 0.20 μM for mice, respectively), than the mature CFU-E (AFB1 IC_{50}: 12.58 ± 5.4 and 40.27 ± 6.05 μM), irrespectively of animal species, (iii) regarding AFM1, a species- and lineage-related susceptibility similar to that observed for AFB1 and (iv) lack of effects after AFB1 *in vivo* treatment on the proliferation of haematopoietic colonies.

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1. Introduction

In recent years there has been an increasing interest in both industry and regulatory bodies concerning the development and the validation of *in vitro* tests able to predict *in vivo* haemato- and myelotoxicity (Parchment, 1998).

Accumulating evidence suggest that the haematopoietic organs are readily affected by a wide variety of drugs and chemicals which may affect the haematopoietic cell renewal system at different stages interfering with cell proliferation and differentiation (Amess, 1993).

Haematopoiesis is a complex interplay between intrinsic genetic pathway of blood cells and their environment, regulated by cytokines. Under appropriate stimulations of these environmental regulatory proteins, erythroid and myeloid stem cells can give rise *in vitro* to phenotypically distinct colonies of differentiated cells. Colony development has been previously used as endpoint in *in vitro* studies of several xenobiotic myelotoxicity, as described in recent pharmacology reviews on alternative *in vitro* test systems (Stephenson et al., 1971; Tepperman et al., 1974; Parchment, 1998; Parent-Massin, 2001; Pessina et al., 2001, 2005; Gribaldo 2002; Malerba et al., 2004). *In vitro* clonogenic assays sufficiently predictive for *in vivo* effects could play a pivotal role in bridging the gap between preclinical toxicology studies in animal models and clinical investigations, and help in the human risk assessment connected with food additives and environmental xenobiotics (Pessina et al., 2002).

Myelosuppression and immunosuppression are common adverse effects observed in toxicological syndromes caused by the ingestion of mycotoxins (Sharma, 1993; Tung et al., 1975; Dugyala et al., 1994).

Mycotoxins, secondary metabolites of fungi that grow on feedstuffs consumed by animals and man, have been found to induce signs of toxicity in different mammalian species. The occurrence of mycotoxins is considered to be a major risk factor affecting human and animal health (Fink-Gremmels, 1999).

Among the groups of mycotoxins considered of major concern for human health, aflatoxins (AF) are both acutely and chronically toxic to mammals, being responsible for acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects (Pitt, 2000). Among the four main naturally produced aflatoxins,

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including aflatoxin B1, B2, G1 and G2, the AFB1 has been shown to cause immunosuppression (Corrier, 1991; Bondy and Pestka, 2000; Oswald et al., 2005).

In Europe, current strong regulation (EC, 2006, 2007) prevents the outbreaks of aflatoxicosis in humans and animals. However, a matter of concern remains the contamination of milk and dairy products by the hydroxylated metabolite of AFB1, the AFM1, excreted from hepatic metabolism (Neal et al., 1998; Fink-Gremmels, 1999).

The Joint FAO/WHO Expert Committee classifies AFB1 as a human carcinogen and proposes no safe dose. Although AFM1 is less carcinogenic (2–10% of potency) than AFB1, it is also a health danger, because this metabolite can survive pasteurization. It has comparable liver toxicity, can reduce the immunological functions in infants, and it is considered to be a human carcinogen (2B) by the International Agency for Research on Cancer (IARC, 1993, 2002) (Eaton and Gallhager, 1994; Chen et al., 2005). This is supported by a number of epidemiological studies demonstrating a positive association between dietary aflatoxins and liver cancer.

Immunosuppression caused by aflatoxin B1 has been demonstrated in various livestock species as well as in laboratory animals (Sharma, 1993). Ingestion of these mycotoxins has been associated with a wide range of adverse effects: e.g. acute mortality, stunted growth, acute hepatic failure and also impaired immunity, with a decreased host resistance to infectious disease (see Corrier, 1991 for a review). Furthermore, ingestion of aflatoxins has also been associated with several diseases such as leukopoenia, anaemia, bone marrow aplasia and myelosuppression (Tung et al., 1975; Dugyala et al., 1994; Chen et al., 2005).

Bone marrow (BM), with its rapidly renewing cell populations, being one of the most sensitive tissue to cytotoxic agents (Bloom, 1993), plays a pivotal role in the immune functions and the toxins, that are able to damage BM cells, may profoundly alter immune response (Dugyala et al., 1994).

Previously, the myelotoxicity of aflatoxins has been demonstrated in animals by assessing bone marrow cells cellularity, microscopic observations, or chromosomal aberrations associated with crude extracts administrated in acute and repeated doses (Barta et al., 1990; Ito et al., 1989).

Moreover, *in vitro* studies performed on myeloid progenitor cells, derived from mice, showed a concentration-dependent decrease in myeloid progenitors after AFB1 exposure (Cukrova et al., 1991; Dugyala et al., 1994).

Due to the relevance of aflatoxins to human health, the present study aimed at (i) comparing, *in vitro*, the sensitivity of human *vs.* murine haematopoietic committed progenitors to AFB1 and its metabolite AFM1, by means of the CFU-GM (myeloid progenitors), BFU-E and CFU-E (erythroid progenitors) assays, and (ii) assessing whether a single *in vivo* treatment with different doses of AFB1 alters the ability of murine multipotent BM cells to form myeloid and erythroid colonies *in vitro*, with the ultimate goal to compare the *in vitro* results with the *in vitro ex-vivo* data, to fully assess different species-related sensitivities.

2. Materials and methods

The experimental design of the present study considered two different steps.

(1) *An in vitro study*: human BM cells (from Poietic Technologies) and murine BM cells (from control mice) were exposed *in vitro* to several concentrations of AFB1 and AFM1 (0.001–50 μg/ml) in clonogenic cultures for different hematopoietic progenitors, e.g. CFU-GM (Colony forming unit – Granulocyte Macrophage), BFU-E (Burst forming unit – Erythroid) and CFU-E (Colony forming unit – Erythroid); the doses have been derived from previous study by Cukrova et al. (1991) and Dugyala et al. (1994).

(2) *An in vitro ex-vivo study*: BM cells, isolated from the femura of mice, given *in vivo* a single i.p. injection of AFB1 (or vehicle = control), were cultured for the appropriate time and finally CFU-GM, BFU-E and CFU-E colonies were scored.

The *in vivo* acute test doses have been selected on the basis of previous studies showing a slight increase in chromosome aberrations in mouse BM after a single dose of 1 mg AFB1/kg body weight, with no increase in micronuclei (Anwar et al., 1994), and, on the contrary, a significant dose-dependent induction of micronuclei after i.p. administration of 2–8 mg AFB1/kg body weight in the rat (Raj et al., 2001).

2.1. Toxins

AFB1 and AFM1 were obtained from Sigma–Aldrich S.r.l., Milan, Italy.

For the *in vitro* experiments, stock solutions were prepared by dissolving AFB1 and AFM1 in acetone (AFB1 = 10 mg/ml acetone; AFM1 = 0.2 mg/ml acetone). Stock solutions were then diluted with Iscove's Modified Dulbecco's Medium (IMDM, Gibco Life Technologies Italia S.r.l., San Giuliano Milanese, Italy) to reach the final concentrations.

For the acute *in vivo* treatment the AFB1 was dissolved in ethanol: corn oil (5%: 95%) just before the injection.

2.2. Murine bone marrow cells

2.2.1. Animals

All experimental procedures involving animals were performed in compliance with the European Council Directive 86/609/EEC on the care and use of laboratory animals.

Male CD-1 mice (3 weeks-old, weighing 14–16 g) were purchased from Charles River Italia (Calco, Italy) and allowed to acclimatize for at least two weeks before the exposure. Throughout the experimental time, animals were kept in an artificial 12 h light: 12 h dark cycle with humidity at 50 ± 10%. Animals were provided rat chow (4RF21 diet) and tap water ad libitum.

2.2.2. Isolation of murine bone marrow cells

This procedure was performed in rigorously sterile conditions on mice belonging to the different experimental groups specifically indicated in (i) Section 2.2.3 for the *in vitro* assay (treatment of cells obtained from control animals) and (ii) Section 2.2.4 for the *in vivo* exposure. Following animal sacrifice by cervical dislocation, intact femura were isolated by cutting muscle ligaments, cleaned, and placed in 100-mm Petri dishes containing 10 ml ice-cold IMDM supplemented with antibiotics (Penicillin 100 U/ml and streptomycin 100 μg/ml; Sigma–Aldrich S.r.l., Milano, Italy). The ends of each femur were cut just below the head and BM was flushed with 3 ml of IMDM without antibiotics. A single-cell suspension was produced by gently and repeatedly drawing the BM cells through a syringe fitted with a 23-gauge needle. BM cells were then filtered through a 100 μm cell strainer and washed by centrifugation at 2500 rpm for 10 min at 20 °C. Then, the pellet was resuspended in IMDM + 30% fetal calf serum (FCS), counted in a Bürker [10 μl of cell suspension was diluted with 90 μl of Hypotonic Liquid (Carlo Erba, Italia)]. Viability was usually 95% or greater, with no differences between experimental groups. The final cell suspension was adjusted to achieve the cell density required for the assay to be performed: (i) 3.0×10^6 cells IMDM + 30% FCS for BFU-E and CFU-E, and (ii) 1.5×10^6 cells IMDM + 30% FCS for CFU-GM.

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2.2.3. Murine BFU-E/CFU-E and GM-CFU assay: in vitro experiment

Murine progenitor cells, collected as previously described, were washed, diluted in 30% FBS–IMDM, and then seeded in MethoCult-M3334 (StemCell Technologies, Vancouver, BC, Canada) for the BFU-E/CFU-E assay or in MethoCult-M3001 (StemCell Technologies, Vancouver, BC, Canada) for the GM-CFU assay. These media are specific for murine cells; the first one contains Iscove's methylcellulose (1%), FBS (15%), BSA (1%), bovine pancreatic insulin (10 μg/ml), human transferrin iron-saturated (200 μg/ml), 2-mercaptoethanol (10^{-4} M), and glutamine (2 mM). Stimulation of the erythroid lineage was obtained by the addition of erythropoietin (3 U/ml). The latter MethoCult-M3001 contains all the components of the previous medium with the addition of CSF (10 ng/ml) to stimulate GM-CFU growth, and lacks 2-mercaptoethanol.

The clonogenic assay was then performed by adding 100 μl of toxin solutions (in IMDM) and 300 μl of cells (3.0×10^6 cells/ml for the erythroid progenitors, or 1.5×10^6 cells/ml for the myeloid progenitors, respectively) directly to a 3 ml methylcellulose tube. The final concentrations range of AFB1 and AFM1 was 0.001–50 μg/ml. Finally, in order to obtain a triplicate for each dose, 1 ml methylcellulose-cell suspension was seeded in 35-mm dishes, and the cultures were incubated at 37 °C in 5% CO_2 for 3 and 10 days (BFU-E/CFU-E cultures) or 7 days (GM-CFU cultures).

2.2.4. Murine BFU-E/CFU-E and GM-CFU assay: acute in vivo treatment

Six mice per dose level received, respectively 0.3, 1, or 3 mg AFB1/kg body weight by single i.p. injection, while control animals were treated with the vehicle only (corn oil). Mice were euthanized by cervical dislocation 48 after the treatment, and blood samples were analyzed from haematological indices. The time elapsed after injection (i.e. 48 h) was chosen based on previous data by Cukrova et al. (1992b) showing *in vivo* AFB1 effects on BM cell growth 2 days after treatment. Additionally, the mean body weight gains were calculated for each experimental group, along with the assessment of selected organ-to-body weight ratios. BM cells were isolated and cultured following the procedure described in the previous Sections 2.2.2 and 2.2.3, obviously with the unique exception that no toxins were added to the cell cultures.

2.3. Human bone marrow cells

2.3.1. Source of human progenitor cells

As the source of progenitors for the CFU-assays, human mononucleated BM cells were used. The cells were obtained frozen, from Poietic Technologies, Inc. (Gaithersburg, MD, USA) and thawed before using. Briefly, 1 ml of cells was rapidly thawed in a water bath at 37 °C and diluted in 1 ml of 2.5% human albumin (Sigma–Aldrich S.r.l., Milano, Italy), IMDM (Gibco Life Technologies Italia S.r.l., San Giuliano Milanese, Italy) 0.22 μm filtered solution, and 10% Fetal Calf Serum heat-inactivated (FCS, Sigma–Aldrich S.r.l., Milano, Italy), 10000U DNase/ml (Sigma–Aldrich S.r.l., Milan, Italy) IMDM solution. After 10 min, the solution was centrifuged at 1200 rpm for 15 min at 18–20 °C. The pellet was then diluted in 30% FCS–IMDM, counted in a Bürker (90 μl Hypotonic Liquid (Carlo Erba, Italia) + 10 μl cell suspension) (cell viability was 90 ± 5%), and used for the clonogenic test at a concentration of 1.5×10^6 cells/ml.

2.3.2. Human BFU-E/CFU-E and CFU-GM assay

BM cells, thawed as previously described, were seeded in MethoCult-H-4330 (StemCell Technologies, Vancouver, BC, Canada) for the BFU-E/CFU-E assay or in MethoCult-H4001 (StemCell Technologies, Vancouver, BC, Canada) for the CFU-GM assay. The MethoCult-H-4330 medium was minus colony-stimulating factor (CSF), but contained FBS (30%), bovine serum albumin (BSA, 1%), methylcellulose (1%), 2-mercaptoethanol (10^{-4} M), glutamine (2 mM) and 3U/ml erythropoietin.

The MethoCult-H4001 medium, without mercaptoethanol, contains CSF, methylcellulose (1%), FBS (30%), BSA (1%), 2-mercaptoethanol (10^{-4} M), glutamine (2 mM), and GM-CSF (10 ng/ml).

The procedure for the clonogenic assay was similar to that used for murine assays, performed by adding 100 μl of toxins solutions (AFB1 and AFM1 in IMDM) and 300 μl of cells (1.5×10^6 cells/ml) directly to a 3 ml methylcellulose tube. Similar were also the AFB1 and AFM1 final concentration tested. In order to obtain a triplicate for each dose of aflatoxins, 1 ml methylcellulose-cell suspension was seeded in 35-mm dishes, and the cultures were incubated at 37 °C in 5% CO_2 for 7 and 15 days.

2.4. Colony scoring

2.4.1. Erythroid lineage

CFU-E and BFU-E were scored after 7 and 15 days of incubation, for human cells, and after 3–5 and 8–10 days, for murine cells, respectively, using an inverted microscope (Zeiss, Axiovert 25) with magnification × 25.

2.4.2. Myeloid lineage

The human CFU-GM colonies were scored after 14 days of incubation, whereas murine CFU-GM were scored after 7 days of incubation, using an inverted microscope (Zeiss, Axiovert 25) with magnification × 25.

The criteria adopted for the identification of the different colony types (immature/mature erythroid or myeloid) have been previously reported by Pessina et al. (2001) and Malerba et al. (2002).

2.5. Data analysis

Cell proliferation is expressed as a percentage of growth, with 100% corresponding to the number of colonies in the vehicle dishes. Colony formation linearity was used as acceptance criteria for colony growth in control dishes (data not shown). The IC_{50} values (concentration of toxin necessary to reduce by 50% the cells viability) were calculated from concentration–response curves in which percent inhibition was plotted against the natural logarithm of the molar toxin concentration. Data will be expressed as mean ± SD of at least three experiments, each carried out in triplicate. Statistical analyses were performed by ANOVA followed by Dunnett's post hoc test. Values of $p < 0.05$ were considered statistically significant.

3. Results

The toxic effects of AFB1 and its metabolite AFM1 on the formation and on the proliferation of erythroid colony forming unit (CFU-E), erythroid burst forming unit (BFU-E) and granulocyte/macrophages colony forming unit (CFU-GM), from human and murine BM progenitors, were evaluated after *in vitro* and *in vitro-ex-vivo* (murine) exposure to the two toxins.

3.1. Murine data: In vitro treatment

Regarding to the *in vitro* study, AFB1 and AFM1 concentration–response curves obtained in three different assays on murine erythroid and myeloid progenitor cells are shown in Fig. 1a, b and c. As clearly detectable observing the dose–response curves, both toxins reduced the colony formation in a concentration-dependent fashion, with an overt toxicity particularly evident in BFU-E and CFU-GM at the highest dose (Fig. 1a and c; Table 1).

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The IC_{50} values calculated for CFU-E, BFU-E and CFU-GM (Table 1) clearly demonstrated that AFB1 seemed to affect more markedly both the myeloid lineage (CFU-GM) and the immature erythroid progenitors (BFU-E) (IC_{50} values of 11.08 ± 2.92 and 1.81 ± 0.20, respectively), than the more mature CFU-E (IC_{50} = 40.27 ± 6.05). In particular, the BFU-E are the most affected colony types (Fig. 1a) in that the dose of 50 μg AFB1/ml was strongly toxic, those of 2, 5 and 10 μg AFB1/ml were markedly suppressive, (toxicity values of 50–80%), and even the lower doses (from 0.001 to 0.1 μg AFB1/ml) significantly inhibited the BFU-E proliferation processes (about 20%).

A similar trend of colony proliferation inhibition was induced by AFM1 in CFU-E, BFU-E and CFU-GM, although in a lesser extent manner (see IC_{50} values in Table 1).

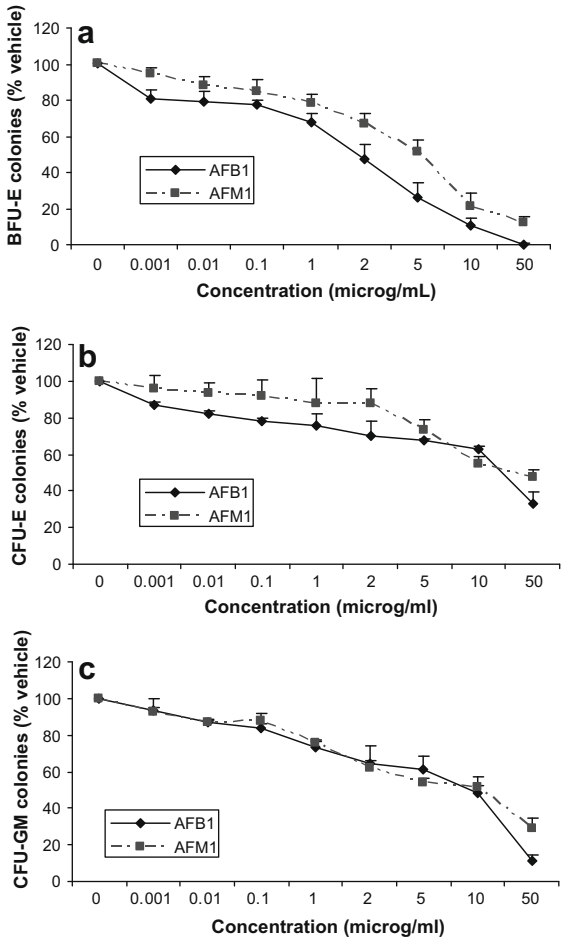

Fig. 1. Concentration-dependent inhibition of (a) BFU-E, (b) CFU-E and (c) CFU-GM colony formation, resulting from *in vitro* exposure of murine bone marrow cells to AFB1 (0.001–50 μg/ml) and AFM1 (0.001–2 μg/ml) toxins. Data are expressed as percent of control colonies and represent the mean ± SD of three independent experiments performed in triplicate.

Table 1
Comparison of IC_{50} values (μM) of human and murine BFU-E, CFU-E and CFU-GM colonies after direct *in vitro* exposure to AFB1 (0.001–50 μg/ml) and AFM1 (0.001–2 μg/ml) . IC_{50} values are expressed as mean ± SD. Minimum of three experiments was carried out in triplicate.

	IC_{50} (μM)	
	Human	Murine
Haematopoietic colonies	*AFB1*	
BFU-E	1.82 ± 0.8	1.81 ± 0.20
CFU-E	12.58 ± 5.4*	40.27 ± 6.05
CFU-GM	2.45 ± 1.08*	11.08 ± 2.92
	AFM1	
BFU-E	2.01 ± 0.9	3.9 ± 2.18
CFU-E	15.7 ± 3.81*	60.5 ± 10.05
CFU-GM	2.40 ± 1.02*	11.5 ± 2.08

* $p < 0.05$ (comparing the same cell type in human *vs.* murine).

3.2. Human data: in vitro treatment

Fig. 2 shows the proliferation inhibition of human erythroid (Fig. 2a and b) and myeloid (Fig. 2c) colonies caused by both AFB1 and AFM1 toxins in a dose-dependent manner, demonstrating a similar trend of toxicity observed in mice. In particular the IC_{50} values indicated that the myeloid progenitors (CFU-GM) and the immature erythroid progenitors (BFU-E) were about 5 and 10 times respectively more sensitive than the respective more mature CFU-E to AFB1 (Table 1).

With respect to the myeloid lineage (Fig. 2c), the most affected colony type, the doses of 10 and 50 μg AFB1/ml were totally toxic, the dose of 5 μg AFB1/ml was strongly suppressive, and the 2 μg AFB1/ml dose still caused 50% inhibition.

Exposure to AFM1 caused similar results to those obtained by AFB1 for all treated colony types.

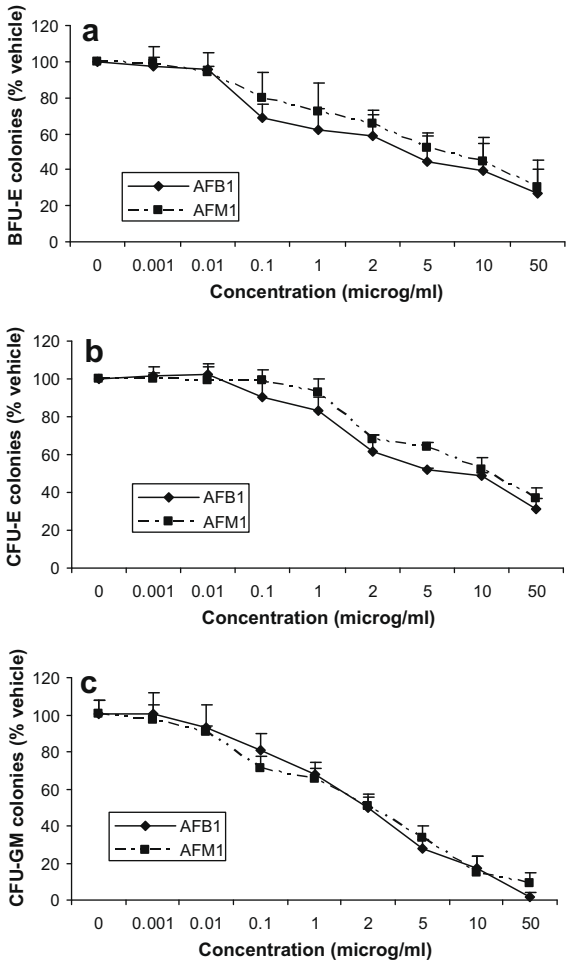

Fig. 2. Concentration-dependent inhibition of (a) BFU-E, (b) CFU-E and (c) CFU-GM colony formation, resulting from *in vitro* exposure of human bone marrow cells to AFB1 (0.001–50 μg/ml) and AFM1 (0.001–2 μg/ml) toxins. Data are expressed as percent of control colonies and represent the mean ± SD of three independent experiments performed in triplicate.

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3.3. Human vs. murine in vitro data

First, our investigation clearly demonstrated that human BM progenitors were more sensitive (about 4 times) than murine BM cells to both toxins, especially for CFU-E and CFU-GM.

Second, irrespectively of the animal species (human or murine), both AFB1 and AFM1 affected more markedly both the myeloid lineage (CFU-GM) and the immature erythroid progenitors (BFU-E), than the more mature CFU-E colonies (Figs. 1 and 2, Table 1).

3.4. Murine data: in vitro ex-vivo experiment

Regarding the *in vitro* formation of CFU-E, BFU-E and CFU-GM colonies after acute *in vivo* exposure to AFB1 (single i.p. administration 0.3, 1, 3 mg/kg b.w.), the toxin neither causes overt toxicity on erythroid lineage nor induces significant myelotoxic effects at any dose (data not shown).

Table 2 reports the results of the haematological parameters measured 48 h after treatment. There were no significant alterations of erythrocyte and leukocyte counts in that the values were within the physiological range at any utilized dose. Similarly, at sacrifice, no significant body weight gain was registered (Fig. 3); furthermore no observable effects were seen in weight of liver, spleen and thymus (Fig. 3). There were no changes in the liver-to-body weight ratio for any of the treatment as also deductible from Fig. 3 (data not shown).

4. Discussion

The present study clearly evidences the *in vitro* toxic effects of aflatoxins, namely AFB1 and AFM1, on myeloid and erythroid human and murine committed progenitors. In particular, comparing *in vitro* human and mice data, both toxins (i) displayed similar dose-dependent toxic effects on both species stem cells, with a stronger sensitivity of the human with respect to the murine BM progenitors, and (ii) affected more markedly the myeloid and the immature erythroid lineage irrespectively of the animal species (human or murine).

Regarding our *in vitro ex-vivo* findings, they seemed to demonstrate that a single i.p. administration of AFB1 (0.3, 1 and 3 mg/kg b.w.) did not cause overt toxicity on erythroid lineage nor induces significant myelotoxic effects at any utilized dose, and furthermore no significant alterations of haematological parameters were observed 48 h after the treatment.

Previous *in vivo* data clearly demonstrated the granulopoietic toxicity of AFB1 in 5–6 week-old male Fischer rats given single i.p. injections of 1 mg/kg (=1/5 of LD_{50}) or 0.1 mg/kg (=1/50 of LD_{50}), followed by a successive rescue reaction (Cukrova et al.,

Table 2
Blood parameters and leukocyte formula of the CD1 mice at the day of sacrifice, 48 h after acute *in vivo* treatment with AFB1 (0.3, 1 and 3 mg/kg b.w.). Data are expressed as the mean ± SD of a single experiment ($n = 6$ *per* experimental group) performed in triplicate.

	AFB1 dose (mg/kg b.w.)				Physiological ranges
	0	0.3	1	3	
Blood parameters					
Erythrocytes (10^6 μl)	5.17 ± 1.77	6.77 ± 0.53	6.11 ± 2.24	6.58 ± 2.30	7–12.5
Leukocytes (10^3 μl)	7.08 ± 4.07	5.68 ± 1.75	4.93 ± 2.43	6.13 ± 1.88	6–15
Haemoglobin (g/l)	9.28 ± 2.02	11.50 ± 2.99	10.47 ± 3.58	11.37 ± 3.07	10–16.6
Haematocrit (%)	29.40 ± 7.99	36.00 ± 10.58	31.17 ± 11.84	34.50 ± 11.55	39–49
Leukocyte formula					
Neutrophyls (%)	17.00 ± 10.39	24.40 ± 10.53	31.00 ± 11.83	36.00 ± 8.85	10–40
Lymphocytes (%)	58.40 ± 7.99	66.60 ± 8.17	61.33 ± 13.13	53.67 ± 2.94	55–95
Eosinophyls (%)	0.67 ± 0.52	0.80 ± 0.84	2.33 ± 2.16	1.67 ± 1.37	0–4
Basophyls (%)	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.41	0.00 ± 0.00	0–0.5

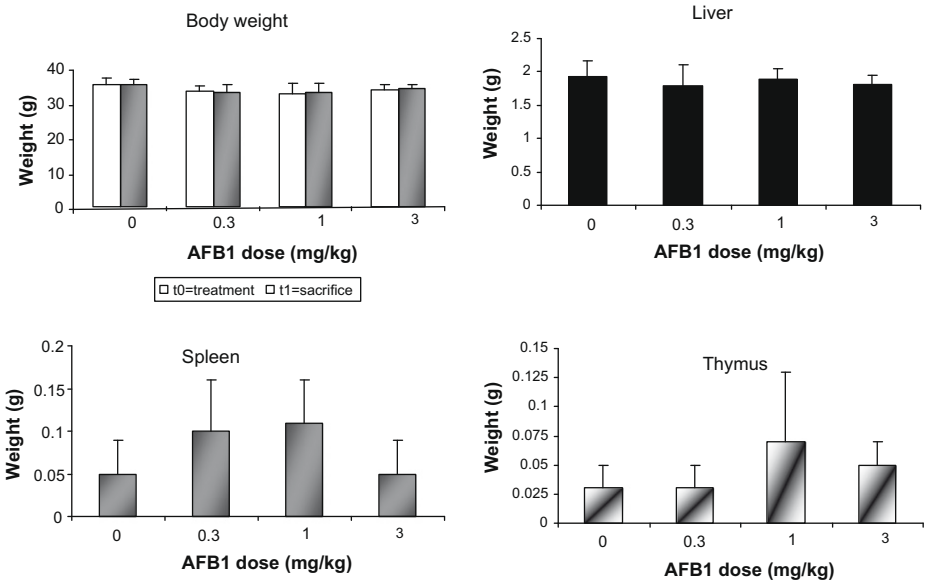

Fig. 3. Histograms showing body weight gain and different organ weight of the CD1 mice exposed to different doses of AFB1 (0.3, 1 and 3 mg/kg b.w.) at the day of sacrifice (48 h after the single i.p. injection). Data are expressed as the mean ± SD of a single experiment ($n = 6$ *per* experimental group) performed in triplicate.

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1992a,b). Dugyala et al. (1994) reported that male CD-1 mice administered 0.03, 0.145 and 0.7 mg/kg b.w. of AFB1 for 2 weeks on alternate days displayed a suppression in granulocyte, macrophage and granulocyte–macrophage (GM) colonies at the highest dose. The mutagenic effects of AFB1 were previously described by Barta et al. (1990), demonstrating a long-term presence of chromosomal aberrations in hamsters and monkeys even after a single i.p. exposure to 0.1 μg–5 mg/kg.

Other experimental studies on the carcinogenicity of AFB1 demonstrated a particularly wide variation in sensitivity in mice with a TD_{50} > 70 μg/kg b.w. per day in C3H and C57BL mice, >5300 μg/kg b.w. per day in Swiss mice, and some strains of mice showing no response at doses up to 150000 μg/kg diet (The Efsa Journal, 2007).

The lack of myelotoxicity found in our investigation may be ascribed at first to the short time of exposure to the toxin *in vivo* (48 h, until the sacrifice); as previously stated, alterations in granulopoiesis have been reported in mice after a two week-repeated AFB1 treatment (Dugyala et al., 1994). Furthermore, the different species (rats *vs.* mice), the route of administration (i.p. *vs.* orally/gavage), the duration of the treatment (single *vs.* repeated doses), and the evaluation of different endpoints (myelotoxicity *vs.* genotoxicity/mutagenicity) may explain the different rate of susceptibility reported in the above mentioned literature and our data.

Furthermore, it should be also mentioned that, notwithstanding the broad similarities in aflatoxin biotransformation across species, there are some key species differences in rate of metabolism, particularly regarding the affinity for and the catalytic activity of the main enzymes involved, particularly glutathione S-transferase (GSTs). GSTs are the main biosynthetic enzymes involved in AFB1-exo-8,9 epoxide conjugation representing the main detoxification pathway. The order of GSH conjugation to AFB1 among species is mouse > rat > human, with humans exhibiting comparatively low conjugation, and, consequently a higher susceptibility (Raney et al., 1992; Kirbi et al., 1993, 1994). Additionally, several *in vivo* studies, using different rodent species, further showed the lowest susceptibility of mice, evaluated by the determination of AFB1-DNA levels and AFB1-albumin adducts (Wild et al., 1996; The Efsa Journal, 2007).

Indeed, our *in vivo* findings seem to be in line with the above explain marked resistance of mice to AFB1, probably due to their high constitutive levels of a hepatic alpha class of GST, with a high affinity for the AFB1-8,9 epoxide, giving them a pronounced detoxification power.

Contrarily to the *in vivo* data, our *in vitro* investigations in mice assessed that both AFB1 and AFM1 caused a dose-dependent inhibition of the growth of erythroid and myeloid committed progenitors, affecting more markedly the myeloid (CFU-GM) and the immature erythroid lineage (BFU-E) than the more mature CFU-E colonies.

The trend of toxicity observed in our murine *in vitro* experiments is in agreement with several data available in literature. Cukrova et al. (1991) demonstrated *in vitro* a concentration-dependent (0.1, 0.5, 1, 5, 10 μg AFB1/ml) suppression of granulopoiesis according to the results of the CFU-GM assay, with the concentrations of 5 μg/ml and 0.5 μg/ml, being these two doses highly and slightly suppressive, respectively. BM cells from control CD-1 mice cultured *in vitro* with 1–50 μM AFB1 also displayed a suppression of all the three types of colonies (Dugyala et al., 1994).

Evaluating our murine data for AFB1 toxicity, obtained with the two different experimental approaches (*in vitro vs. in vivo*), the *in vitro* IC_{50} data (Table 1) underscore the moderate sensitivity of murine cells, with special reference to CFU-GM and CFU-E, in line with the poor susceptibility of mice to AFB1 myelotoxic effects *in vivo*. As above hypothesized, the differences among *in vivo* and *in vitro* data may be ascribed to the short time of *in vivo* exposure to the toxin; noteworthy, in *in vitro* experiments the myeloid and erythroid committed progenitors were continuously exposed to AFB1 throughout all the culturing period until the day of scoring (from minimum 3 to maximum 10 days).

In humans cultures, the inhibition of proliferation of erythroid and myeloid colonies displayed a dose-dependent toxicity similar but, markedly, more pronounced to that observed in murine cells.

In particular, the specific IC_{50} values for each type of stem cells were about 4 times lower in humans than in mice.

Moreover, AFB1 IC_{50} values clearly demonstrated that the myeloid progenitors (CFU-GM) and the immature erythroid progenitors (BFU-E) were about 5 and 10 times respectively more sensitive than the respective more mature CFU-E to AFB1; a similar trend of toxicity was induced by AFM1 in all human colony types, as clearly deductible from IC_{50} values.

Notably, our *in vitro* findings regarding AFM1, in both human and murine cells, seem to be in line with previous literature data, showing that, similarly to AFB1, the conjugation of AFM1-epoxide with reduced GSH, representing the main detoxification pathway, is catalyzed by mouse but not human liver cytosol (Neal et al., 1998).

Taken together, our *in vitro* findings could be probably explained by (i) the lack of a detoxification pathway in both murine and human myeloid and erythroid progenitors cultured *in vitro*; (ii) the poor resistance of human cells to AFB1 and AFM1, probably due to the scarce detoxication pathway typical of human biotransformation metabolism of these toxins.

An overall evaluation of our findings clearly indicate (i) the need of an integrated study strategy to rightly evaluate the toxic potential of a toxin, taking into careful consideration the species-related differences and (ii) the need of caution in designating the metabolic conversion of AFB1 to AFM1 as an essentially detoxification process.

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